APPENDIX C

In Vitro Evaluation of Anticoagulant Activity of Enoxaparin Fractions

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In vitro evaluation of anticoagulant activity of Enoxaparin fractions

Summary

This study was performed to compare the anti-factor Xa- and anti-factor IIa activity of six 1,6-anhydro fractions of Enoxaparin with different saccharide chains (Hexasaccharides, Octasaccharides, Decasaccharides, Dodecasaccharides, < 16-mer, ≥ 16-mer) with their corresponding non-anhydro counterparts. Additionally, the anticoagulant effect of both sets of different fractions was assessed by thromboelastography. The Non-Anhydro hexasaccharides and ≥16-mer fractions displayed higher specific aFXa- and aFIIa- activity than the Anhydro-counterpart. In thromboelastography, at the same aFXa-concentration, the >16-mer "Non-Anhydro"had a higher anticoagulant potency than the >16-mer "Anhydro".

Study objectives

The aim of this study was to evaluate the anti-factor Xa activity (aFXa) and the anti-factor IIa (aFIIa) of six 1,6-anhydro fractions of Enoxaparin with different saccharide chains (Hexasaccharides, Octasaccharides, Decasaccharides, Dodecasaccharides, < 16-mer, ≥ 16-mer) and their corresponding non-anhydro counterparts. Moreover, the anticoagulant effect of both sets of different fractions was assessed by thromboelastography.

Material and Methods

The following different fractions of Enoxaparin, provided by Dr. Christian Viskov, Glycochemistry unit, Paris research center, were analyzed:

Standard Enoxaparin fractions	"0%" Enoxaparin fractions
WSD3093-Hexasaccharides	DIA2844-Hexasaccharides
WSD3093-Octasaccharides	DIA2844-Octasaccharides
WSD3093-Decasaccharides	DIA2844-Decasaccharides
WSD3093-Dodecasaccharides	DIA2844-Dodecasaccharides
WSD3093 < Hexadecasaccharides	DIA2844 < Hexadecasaccharides
WSD3093 ≥ Hexadecasaccharides	DIA2844 ≥ Hexadecasaccharides

The compounds were dissolved in aqua dest at concentration of 1 mg/ml, further dilutions were made in human standard plasma (IL).

Anti-FXa- and Anti-FIIa-activity

AFIIa-activity was performed by in house established microtiter-plate method using chromogenic substrate S-2238, thrombin and human standard plasma (IL) as source for ATIII corresponding to method described by V Putten et al. (2) and CN Berry et al. (3). AFXa activity in human standard plasma was determined with ACL 7000 automated coagulation instrument (Instrumentation Laboratories, IL) using Heparin-kit containing ATIII, FXa and the chromogenic substrate S-2765. Anti-Xa/IIa activity of the above indicated compounds were determined by use of a standard calibration curve constructed with Enoxaparin.

Thromboelastography (TEG)

The principle of TEG is based on the measurement of viscoelastic characteristics of recalcified whole blood sample against the time. Blood clotting is monitored at 37°C in an oscillating plastic cylindrical cup and a coaxially suspended stationary pin. The torque experienced by the pin is plotted as a function of the time. The amplitude on the TEG tracing is a measure of the rigidity of a blood clot. TEG was performed on TEG 5000 instrument (Hemoscope) according to manufacturer's instructions, disposable supplies were purchase from Hemoscope. Briefly, 20 µl 0,2 M CaCl and 10 µl compound (diluted in aqua dest.) were preloaded to a plastic cup, 330 µl citrated whole blood were added and the measurement started immediately after mixing. Based on the anti-factor Xa acitvity plasma concentration of each tested compound was 0.05, 0.075 and 0.1 U/ml. TEG assay results were quantified according to the reaction time a detectable clot was formed (R) and the maximum amplitude (MA) indicating the clot strength.

Statistical Analysis

Data are expressed as means ± SEM. Biometrical evaluation was performed by standard biostatistical analysis using GraphPadPrism (Version 3) or SigmaStat 2.03. The statistical significance of differences between the standard Enoxaparin fractions (Anhydro, WSD) and "0%"-Enoxaparin fractions (Non-Anhydro, DIA) compounds was evaluated by Student's t-Test. Significance was defined at $p<0.05$.

Results

Table 1 – Effect of Enoxaparin fractions on anti-factor Xa activity (U/mg)

	WSD 3093	SEM	DIA 2844	SEM	
Hexasaccharide	9,8	0,7	13,9	0,6	*P<0.05*
Octasaccharide	42,9	4,0	47,6	5,2	*NS*
Decasaccharide	69,3	3,4	67,0	7,5	*NS*
Dodecasaccharide	78,4	6,1	95,1	4,8	*NS*
< Hexadecasaccharide	51,3	5,5	56,9	0,7	*NS*
>= Hexadecasaccharide	140,0	8,0	164,6	8,8	*P<0.05*

Table 2 – Effect of Enoxaparin fractions on anti-factor IIa activity (U/mg)

	WSD 3093	SEM	DIA 2844	SEM	
Hexasaccharide	0,0	0,01	0,1	0,01	*P<0.05*
Octasaccharide	0,1	0,01	0,2	0,01	*NS*
Decasaccharide	0,2	0,01	0,2	0,01	*NS*
Dodecasaccharide	0,2	0,01	0,2	0,01	*NS*
< Hexadecasaccharide	0,1	0,01	0,2	0,01	*NS*
>= Hexadecasaccharide	44,5	2,18	60,3	2,80	*P<0.05*

Table 3 - Effect of Enoxaparin fractions on thromboelastography (parameters reaction time [min], maximum amplitude [mm])

		WSD3093				DIA2844			
	anti-fXa activity	Reaction Time	SEM	Maximum amplitude	SEM	Reaction Time	SEM	Maximum amplitude	SEM
Hexasaccharide	0.1U/ml	56,5	5,2	14,5	4,0	79,1	12,8	14,5	5,3
	0.075U/ml	35,7	3,7	20,0	0,5	34,2	2,4	25,2	1,0
	0.05U/ml	30,8	2,3	28,0	3,4	22,8	0,2	24,0	0,0
Octasaccharide	0.1U/ml	40,8	7,6	14,8	1,4	78,8	25,0	20,2	5,5
	0.075U/ml	32,6	0,3	20,8	0,1	31,1	4,0	24,3	1,8
	0.05U/ml	30,6	2,4	21,8	1,2	24,9	3,8	20,0	0,3
Decasaccharide	0.1U/ml	60,1	9,0	17,0	4,2	50,6	8,6	20,3	0,9
	0.075U/ml	31,1	1,1	20,3	0,3	42,8	6,4	21,0	3,1
	0.05U/ml	81,2	28,5	23,3	1,3	25,0	2,3	21,5	0,2
Dodecasaccharide	0.1U/ml	32,6	6,0	22,5	0,7	39,0	3,1	27,7	2,8
	0.075U/ml	23,2	3,2	20,2	0,1	27,2	2,6	27,3	2,7
	0.05U/ml	32,0	5,8	25,3	2,6	33,5	1,6	21,0	0,2
< Hexadecasaccharide	0.1U/ml	42,4	5,6	20,7	1,7	52,3	12,3	24,2	3,9
	0.075U/ml	35,9	4,4	19,7	0,9	49,0	15,6	24,2	3,0
	0.05U/ml	52,4	6,7	24,0	1,2	28,0	3,2	20,3	0,4
>= Hexadecasaccharide	0.1U/ml	53,3	2,0	19,3	0,3	146,5	10,4	7,5	1,6*
	0.075U/ml	48,5	5,8	19,0	0,3	101,3	11,9	14,8	2,5
	0.05U/ml	36,0	3,7	20,3	1,1	36,5	2,9	19,7	0,3

*P<0.05 (WSD vs. DIA)

Figure 1: – **Effect of Enoxaparin fractions on anti-factor Xa activity (U/mg)**

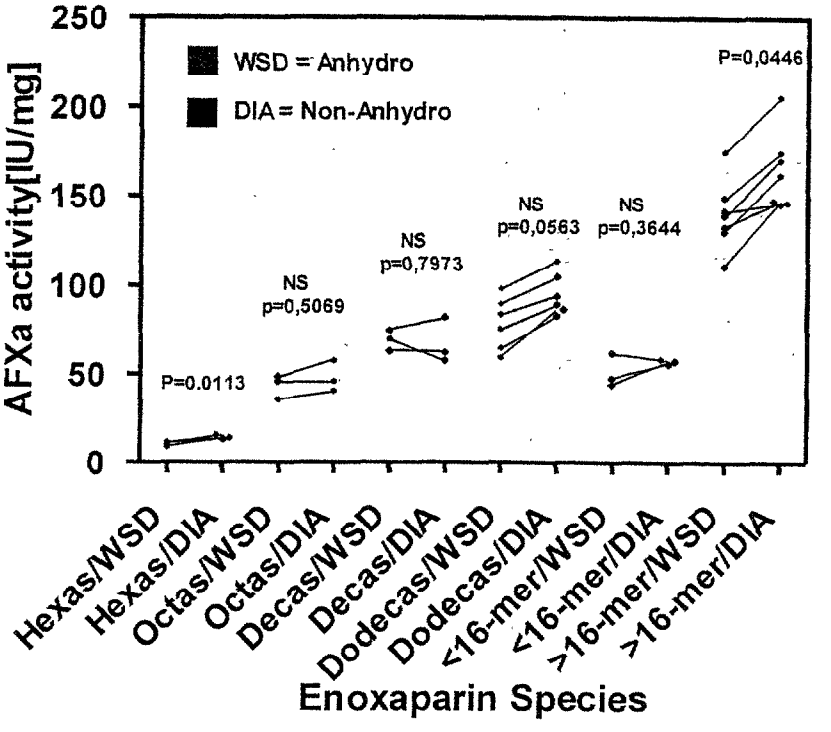


Figure 2: – **Effect of Enoxaparin fractions on anti-factor IIa activity (U/mg)**

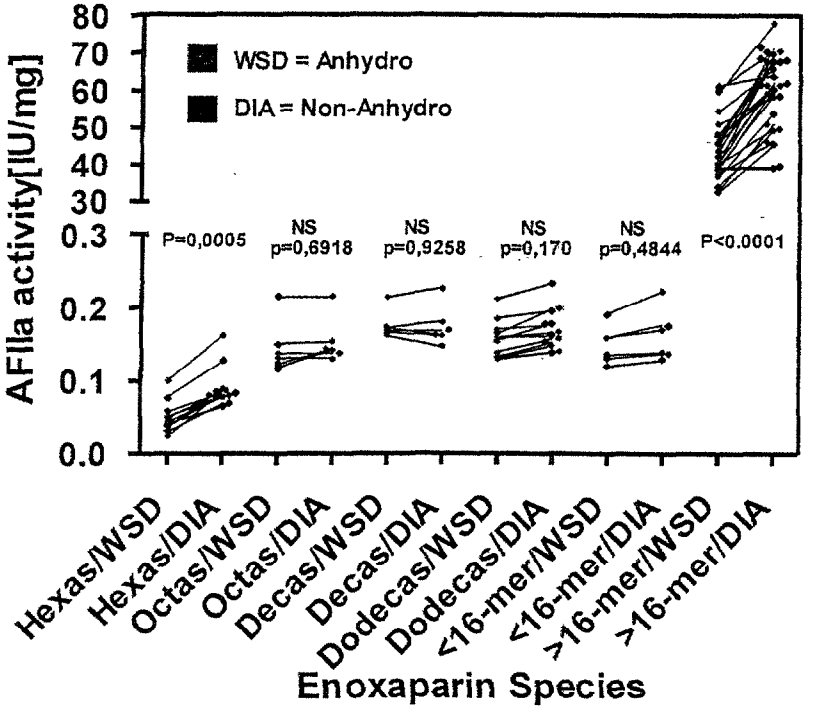


Figure 3: Effect of Enoxaparin fractions on thromboelastography (parameter: reaction time)

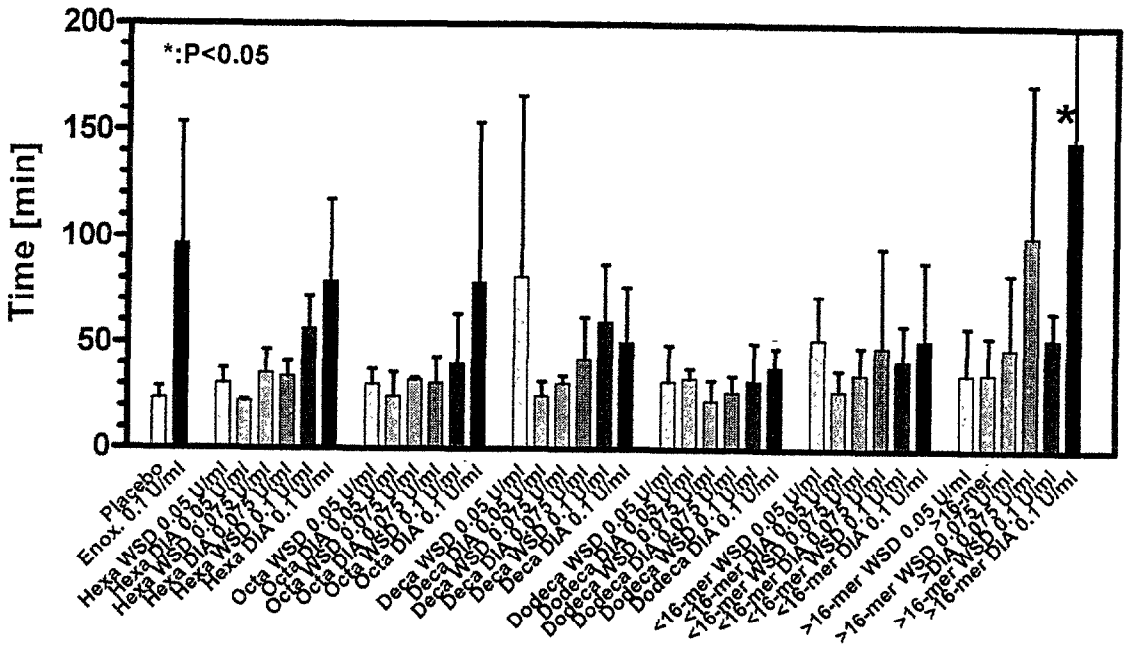
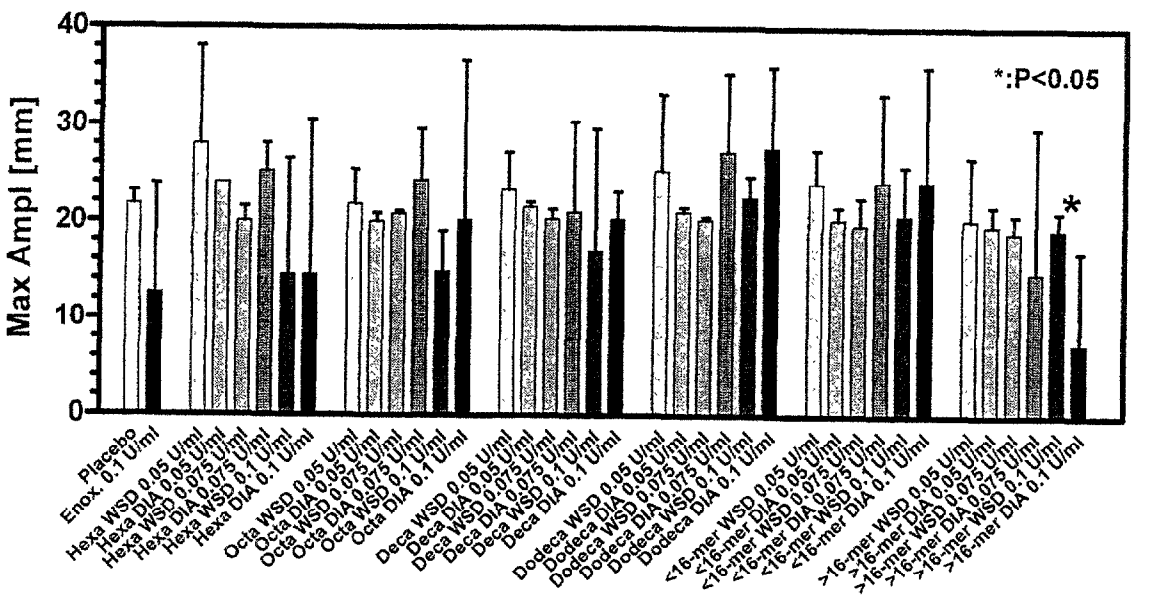


Figure 4: Effect of Enoxaparin fractions on thromboelastography (parameter: maximum amplitude)



Results

Statistically significant differences in aFXa- and aFIIa-activity were found between hexasacharides Anhydro and Non-Anhydro fractions and >16-mers Anhydro- and Non-anhydro fractions.
In both cases the Non-Anhydro fractions displayed higher specific activity.
In the thromboelastography experiments, the Non-Anhydro >16-mer fraction exerted statistically significant higher anticoagulant effects than the Anhydro counterpart. This was observed at the highest concentration of 0.1 aFXa IU/ml for both parameters, reaction time and maximum amplitude.

Conclusion

The aim of this study was to evaluate the anti-factor Xa activity (aFXa), the anti-factor IIa (aFIIa) of six 1,6-anhydro fractions of Enoxaparin with different saccharide chains (Hexasaccharides, Octasaccharides, Decasaccharides, Dodecasaccharides, < 16-mer, ≥ 16-mer) and their corresponding non-anhydro counterparts. Moreover, the anticoagulant effect of both sets of different fractions was assessed by thromboelastography.

The aFXa- and aFIIa-activities were determined to evaluate the potential of the different heparinoid fractions to accelerate the ATIII-induced inhibition of α-thrombin and coagulation factor Xa. The Non-Anhydro hexasaccharides and ≥16-mer fractions displayed higher specific aFXa- and aFIIa- activity than the Anhydro-counterpart.

Thromboelastography was conducted to evaluate the effect of the enoxaparin fractions on the coagulation system. At the same aFXa-concentration, the Non-Anhydro ≥16-mer fraction lead to a significant prolongation of reaction time as well as reduction of clot strength indicated by the maximum amplitude compared to the Anhydro >16-mer fraction. In general, at the same aFXa-concentration, the >16-mer "Non-Anhydro" (DIA) had a higher anticoagulant potency than the >16-mer "Anhydro" (WSD) analyzed by thromboelastography.

Reference

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3 Van-Putten,-J; van-de-Ruit,-M; Beunis,-M; Hemker,-H-C. Automated spectrophotometric heparin assays. Comparison of methods.
Haemostasis. 1984; 14(2): 195-204

I hereby certify that the experimental studies described and the analyses presented in this report were conducted by me and /or my supervision.

Volker Laux
PRINT NAME

SIGNATURE

January 29th, 2004
DATE